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Original Articles

Pneumocystis carinii pneumonia in HIV-infected patients: effect of steroid therapy on surfactant level

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Previous studies have suggested alterations in pulmonary surfactant lipid in the setting of *Pneumocystis carinii* pneumonia in HIV-infected patients. Because pulmonary surfactant lipid is composed of a variety of lipid products and because other phospholipids might be present in bronchoalveolar lavage (BAL) lipid determinations, a single molecular species of phospholipid which comprises a substantial portion of the surfactant lipid fraction, dipalmitoyl phosphatidylcholine (DPPC), was measured by capillary column gas chromatography in BAL samples taken at the time of the diagnosis of *P. carinii* pneumonia, and 10 days after treatment for *P. carinii* pneumonia. DPPC was measured at day 0 and day 10 in seven patients who had been randomized to receive methylprednisolone adjuvant therapy for *P. carinii* pneumonia and in six patients who had been randomized to not receive methylprednisolone therapy. The level of DPPC in BAL from all patients at day 0 was $0{\cdot}49 \pm 0{\cdot}06\,\mu g\,ml^{-1}$ BAL. This level is significantly lower that the level of DPPC determined in BAL from five normal volunteers $2{\cdot}48 \pm 0{\cdot}40\,\mu g\,ml^{-1}$. At day 0, the BAL level of DPPC in patients treated with methylprednisolone was not different from the BAL level of DPPC in patients not treated with methylprednisolone. By day 10 of therapy for *P. carinii* pneumonia, BAL levels of DPPC in all patients had increased to $1{\cdot}05 \pm 0{\cdot}19\,\mu g\,ml^{-1}$ BAL. At day 10 DPPC levels in the methylprednisolone treated group were not different from the group not treated with methylprednisolone. We conclude that in HIV-infected patients, lung surfactant lipid is reduced in the setting of *P. carinii* pneumonia. The lipid levels return toward normal levels with treatment. Adjuvant therapy with corticosteroids does not alter the rate of recovery of surfactant lipid levels at least after 10 days of therapy.

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Introduction

Pneumocystis carinii pneumonia (PCP) is still a common lung disease affecting patients with HIV infection. It can cause a life-threatening pneumonia if therapy is not instituted promptly. The mechanism by which *Pneumocystis* causes lung damage and abnormal gas exchange, however, is not well understood. Possible mechanisms might include alveolar filling, damage to the type I or type II cells in the alveolar space as a direct effect of the organisms or as an effect of activation of macrophages, lymphocytes or fibroblasts as a part of the inflammatory response to the organism. If the pneumonic process results in damage to alveolar type II cells, production of pulmonary surfactant may be altered.

Human pulmonary surfactant is a complex mixture of glycerolphospholipids, other lipids, and surfactant proteins. The composition of the glycerolphospholipids includes primarily dipalmitoyl-phosphadidylcholine (DPPC), other species of phosphatidylcholine, and phosphatidylglycerol (1). Studies investigating the effect of HIV infection and *P. carinii* pneumonia on the lipid and protein component of pulmonary surfactant have suggested abnormalities (2–4). When the total lipid quantity from bronchoalveolar lavage fluid was analysed, variable results were reported (2,3,5). One study reported a reduction in BAL total

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phospholipids and phosphatidylcholine separated by thin layer chromatography and assayed as organic phosphorus. In a separate study, normal volunteers and HIV-infected patients without PCP had significantly higher levels of BAL phospholipid assayed by gas chromatography than patients with a mild episode of PCP or moderate to severe PCP (3). There was a parallel fall in diacylglycerol lipids, suggesting that native surfactant lipid was not merely degraded, but that production was reduced (3). A separate study which utlilized enzymatic assays of phospholipids did not find a decrease in BAL phosphatidylcholine or phospholipid and found an increase in phosphatidylglycerol in BAL fluid of HIV-infected patients with *P. carinii* pneumonia (5). Experimental animal models of *P. carinii* pneumonia also suggest abnormalities of lung surfactant (6,7). Rats treated with dexamethasone who then develop *P. carinii* pneumonia have reduced levels of lung lavage phosphatidylcholine and other phospholipids, while animals treated with dexamethasone who do not develop *P. carinii* pneumonia have higher levels than untreated rats (6). The increase in lung lavage phosphatidylcholine in dexamethasone-treated rats is consistent with the previously reported increase in phosphatidylcholine production by fetal pneumocytes treated with dexamethasone (1). Moreover, the adverse effect of *P. carinii* pneumonia on surfactant lipid production by pneumocytes is suggested by *in vitro* studies of type II pneumocyte function. Isolated alveolar type II cells from rats with *P. carinii* pneumonia incorporate less radiolabelled choline into secreted phosphatidylcholine than do type II cells from rats without *P. carinii* pneumonia (7).

It is postulated that the inflammatory or immune response to the organism is in part responsible for severe abnormalities of gas exchange noted in the setting of *P. carinii* pneumonia. This theory led to the study of adjunctive glucocorticosteroid therapy to prevent or ameliorate this effect (8–11). Though these studies provided evidence of efficacy in terms of decreased hypoxaemia and mortality, and lead to the consensus statement regarding steroid use in PCP (12), the mechanism of this effect is not well understood. As was previously noted, glucocorticosteroid treatment of alveolar type II cells may increase cellular production of surfactant phosphatidylcholine (1). If a decrease in the level of surfactant lipid is a sequela of human *P. carinii* pneumonia, then it seems possible that glucocorticosteroid therapy might preserve lung function by minimizing this loss.

It was the purpose of this project to investigate whether a major molecular species of surfactant lipid, DPPC, is reduced in the setting of *P. carinii* pneumonia and to study whether adjuctive glucocorticosteroid therapy in the setting of *P. carinii* pneumonia increases the quantity of lung surfactant lipid. In order to answer this question, surfactant lipid was extracted and quantitated from bronchoalveolar lavage fluid obtained from HIV-infected patients with *P. carinii* pneumonia who were part of a randomized study measuring the effect of standard therapy plus either methylprednisolone ($2\ mg\ kg^{-1}\ day^{-1}$) or no methylprednisolone treatment (8).

Methods

As part of a large, multicentre study, patients were randomized to receive either methylprednisolone ($2\ mg\ kg^{-1}\ day^{-1}$) or no adjunctive therapy, as described previously (8). Therapy for *P. carinii* pneumonia was started after the first bronchoscopy and bronchoalveolar lavage (BAL). The total volume instilled was that required to obtain a return of approximately 100 ml. The average volume instilled on day 0 was 220 ± 10 ml and on day 10 was 226 ± 13 ml. Average return was $55 \pm 4\%$ ($n=13$) on day 0 and $51 \pm 4\%$ ($n=11$) on day 10. The fluid was transported to the laboratory on ice, centrifuged at $1500 \times g$ for 10 min to remove cells and *P. carinii* organisms and aliquots of supernatant were then frozen at $-20°C$ until studied. BAL from five normal volunteers was processed in the same manner. Aliquots of BAL (0·50–1·0 ml) were centrifuged for 1 h at $82\,000 \times g$ to pellet the surfactant lipid. The lipid pellet was then quantitatively transferred using three aliquots of 1·0 ml of 0·9% saline, and added to 10 μg of C15, C15-PC dried previously under N_2. This solution was then lyophilized. C15, C15-PC is a non-physiological lipid which was used as an internal yield standard for the extraction procedure. The lyophilized material was then extracted using a modified Folch procedure (13) in which the lyophilized lipids were first placed in 10 ml of cold chloroform:methanol (2:1), and then 2·5 ml of distilled H_2O were added. The lipid layer was then removed from under the aqueous layer, percolated through Na_2SO_4, dried under N_2, and resuspended in 50 μl of C/M 2:1.

The lipids were then separated by two dimensional thin layer chromatography (TLC) by the method of Rouser (14) using silica H plates with 7·5% magnesium acetate (Analtech Corp., Newark, Delaware, U.S.A.). The lipids were visualized by spraying with fluoroscein, exposing to fumes from 30% NH_4OH, and then visualizing under ultraviolet light. The PC spots were then removed from the plates, and the lipids re-extracted from the TLC silica using the method of Arvidson (15).

The choline moiety was hydrolysed from the PC to form the equivalent 1,2-diacylglycerol compounds using 10 U of phospholipase C, type I, from *C. perfringens* (3) (Sigma Corporation, St. Louis, MO, U.S.A.) and shaken for 1 h at 40°C. The hydrolysed lipids were extracted by adding 10 ml of C/M 2:1, and the lower, organic layer removed and percolated through sodium sulphate. Two micrograms of Tridecanoin (TDC), the internal standard, were added at this point, and the lipid mixture dried under nitrogen.

The trimethylsilyl derivatives of the lipids were then formed by adding 100 μl Trisyl-BSA, formula P (Pierce Chemicals, Rockford, IL, U.S.A.) and incubating for 1 h at 40°C. The lipids were dried under N_2 and suspended in 100 μl hexane, in preparation for analysis by gas chromatography.

For gas chromatographical analysis, the lipid samples (2 μl each) were injected into a Hewlett-Packard 5880 gas chromatograph (Hewlett-Packard Corporation, Kennett Square, PA, U.S.A.) equiped with an automatic liquid

TABLE 1. Patient characteristics

	All patients (n=13) (± SEM)	Patients receiving methylprednisolone (n=7) (± SEM)	Patients receiving no adjunctive therapy (n=6) (± SEM)
Sex	11 men 2 women	6 men 1 woman	5 men 1 woman
Age (years)	44 ± 3·1	48 ± 4·5	38·8 ± 3·4
P_AO_2 (mmHg)	55 ± 2·9	59·5 ± 2·7	50 ± 5·1
A–a difference (mmHg)	50·7 ± 3·6	50 ± 4·5	51·8 ± 6
CD4 count (cells mm^{-3})	44 ± 16	27·0 ± 10	63·5 ± 33·5
LDH (IU)	766 ± 123	594 ± 130	967 ± 200

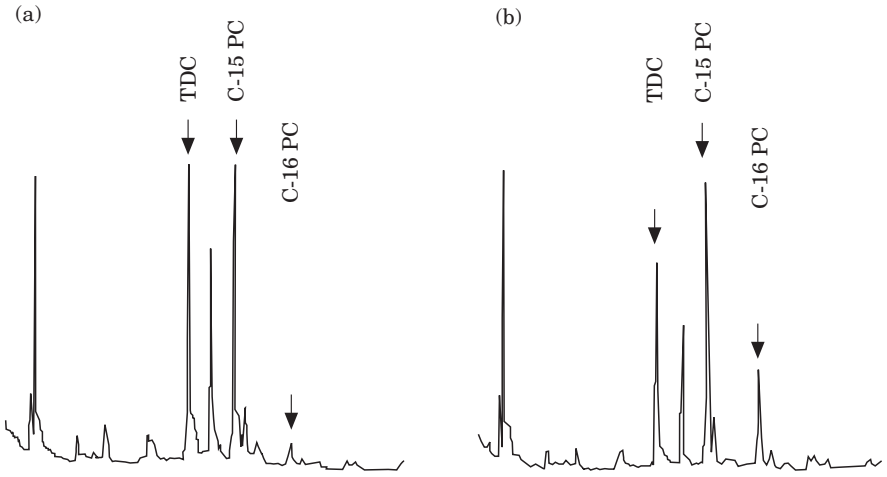

FIG. 1. Representative chromatogram of BAL DPPC of a patient with *P. carinii* pneumonia at day 0 (a) and day 10 (b). See Methods section for details. TDC, tridecanoin; C-15 PC, C-15, C-15 phosphatidylcholine; C-16 PC, dipalmitoylphosphatidylcholine (DPPC).

sampler using an on-column injector. The column employed was a 15-m, 0·25-mm, aluminum-clad capillary column coated with 5% phenyl silicone (Quadrex Corporation, New Haven, CT, U.S.A.), attached to a 2-m, 0·53 mm, uncoated, deactivated guard column (Hewlett-Packard Corporation). The temperature program was as follows:

1. Initial temperature 50°C for 5 min, followed by:
2. 30° min^{-1} to 150°, final time at 150°=0 min;
3. 20° min^{-1} to 230°, final time at 230°=0 min;
4. 10° min^{-1} to 280°, final time at 280°=0 min;
5. 5° min^{-1} to 340°, final time at 340°=30·37 min.

The quantities of both the C15,C15-PC and C16,C16-PC were calculated by comparison to known standards (16). All samples were run blinded.

Statistical analysis of BAL lipid levels in the two groups was done by unpaired t-test in Microsoft Excel. Comparison of the changes in BAL lipid levels from day 0 to day 10 was done by paired-sample two-tailed t-test. $P<0·05$ was considered significant.

Results

Surfactant lipid from matched day 0 and day 10 BAL specimens was extracted from 13 patients (26 specimens in total). The clinical characteristics from these 13 patients are shown in Table 1. Overall, of the 13 patients who had BAL procedures, 11 were men and 2 were women. Seven patients (6 men, one woman) were randomized to recieve methylprednisolone and six patients (five men, one woman) were randomized to receive no methylprednisolone therapy. There were no significant differences between the two groups in terms of age, room air P_AO_2 on admission, alveolar–arterial (A–a) O_2 difference, CD4 count, or serum lactate dehydrogenase (LDH) level on admission.

Representative chromatographs of BAL from a patient with *P. carinii* pneumonia who had BAL performed at day 0 (left panel) and day 10 (right panel) are presented in Fig. 1. The overall yield for the BAL lipid extraction procedure, as measured by C15,C15-PC recovery was 40%.

The levels of dipalmitoyl phosphatidyl choline (DPPC) in BAL expressed in μg DPPC ml BAL^{-1} are shown in Table

TABLE 2. Surfactant lipid levels comparison between methylprednisolone and placebo-treated patients: days 0 to 10

Day	All patients	Patients receiving methylprednisolone	Patients receiving placebo	*P*-value†
0	0·49 ± 0·06*	0·53 ± 0·06	0·45 ± 0·11	n.s.
10	1·05 ± 0·19	1·03 ± 0·29	1·07 ± 0·24	n.s.

*μg DPPC ml BAL^{-1} ± SEM.
†Comparing different patient groups at day 0 or day 10 (n.s., not significant).

TABLE 3. Surfactant lipid levels changes between day 0 and day 10

	Change days 0–10 (μg DPPC ml BAL^{-1} ± SEM)	*P*-value
Patients receiving methylprednisolone	0·50 ± 0·31	0·16
Patients receiving no adjunctive therapy	0·61 ± 0·2	0·02

Results are in μg of C16, C16-PC ml BAL^{-1}.

2. For all patients, at the time of diagnosis of *P. carinii* pneumonia, the measured level of DPPC was 0·49 ± 0·06 μg ml BAL^{-1}. For patients who did not receive methylprednisolone therapy, the level of DPPC at day 0 was 0·45 ± 0·11 μg ml BAL fluid^{-1}. This level was not different from the DPPC level measured at day 0 in patients randomized to receive methylprednisolone adjunctive therapy (0·53 ± 0·06 μg ml BAL^{-1}). By day 10 the average DPPC level had increased to 1·05 ± 0·19 μg ml BAL^{-1} in the group as a whole (P0·02 *vs.* day 0). At day 10, the DPPC level for those who had received methylprednisolone therapy was not different from those who had not (1·03 ± 0·29 *vs.* 1·07 ± 0·24 μg ml BAL^{-1}, respectively) (Table 2). At both time-points, BAL DPPC levels were significantly decreased when compared to surfactant lipid extracted from BAL fluid from five normal volunteers (2·48 ± 0·40 μg ml BAL fluid^{-1}, P<0·05). The changes in BAL DPPC in paired samples between day 0 and day 10 of treatment are presented in Table 3. Within the two groups, the increase in DPPC level from day 0 to day 10 did not reach statistical significance in the methylprednisolone treated patients and was significantly higher in the patients not receiving methylprednisolone therapy (P=0·02). Therefore, levels of DPPC in BAL of patients with *P. carinii* pneumonia are low at the time of diagnosis of pneumonia and return towards normal with treatment of the pneumonia.

Discussion

Despite the advent of effective regimens of prophylaxis, *P. carinii* pneumonia is still a cause of morbitity in HIV-infected patients (17,18). The specific mechanisms by which *P. carinii* pneumonia causes hypoxaemia are not well understood (19). Interstitial inflammation, alveolar exudate and possibly surfactant depletion might all play a significant role. Alterations of pulmonary surfactant lipid, surfactant protein concentration and surfactant activity have been reported in a variety of types of lung injury. In humans, acute lung injury is associated with decreases in BAL phosphatidylcholine and surfactant protein A (20,21). In adults with bacterial pneumonia and in children with bacterial or viral pneumonia, surfactant protein A concentrations may be reduced (22,23). The proinflammatory cytokine tumour necrosis factor (TNF) inhibits the production of surfactant protein A by isolated alveolar type II cells (24,25). In experimental animal models of acute lung injury, alterations in disaturated phosphatidylcholine have been reported in lung injury induced in dogs (26) and in rabbits (27). Similarly, changes in lung lavage phosphatidylcholine have been reported in a rat model of *P. carinii* pneumonia (6).

We utilized a measurement of a single molecular species of phospholipid (DPPC) which is characteristic of pulmonary surfactant in order to assess more specifically any changes in surfactant lipid in the BAL of patients with *P. carinii* pneumonia. At the time of diagnosis of *P. carinii* pneumonia, surfactant DPPC was significantly reduced in all. By day 10 of treatment for *P. carinii* pneumonia, BAL DPPC was significantly higher than at day 0; although, BAL DPPC at day 10 was still lower than that measured in normal volunteers. The measured levels of DPPC from BAL were lower than might have been expected if previous studies of BAL diacylglycerol lipids reflect only surfactant lipid. This difference may be accounted for by losses in additional separation precedures necessary to measure only DPPC or perhaps by the presence of other non-surfactant diacylglycerol lipids in BAL.

While a reduction in BAL phospholipid or phosphatidylcholine in HIV infected patients with *P. carinii* pneumonia has been previously reported (2,3), others have reported no reduction in total BAL phospholipid or phosphatidylcholine (5). The differences in these reports may relate to different assay methods or to differences in processing

the BAL. *Pneumocystis carinii* has been reported to bind surfactant protein A (19). It seems possible that the organism binds to surfactant protein and lipid and that removal of the organism removes surfactant lipid as well.

Corticosteroid adjunctive therapy in patients with *P. carinii* pneumonia appears to modulate the activity of macrophages and lymphocytes participating in the inflammatory response in the lung and thereby prevent a substantial decline in gas exchange during initial therapy (28,29). Given the experimental evidence that corticosteroid therapy is capable of stimulating alveolar type II cell production of phosphatidylcholine, it seemed possible that adjuvant corticosteroid therapy might induce an increase in BAL levels of DPPC. At least at day 10, there was no evidence that adjunctive therapy with glucocorticosteroids made a difference in the level of lung surfactant as measured by BAL DPPC.

The importance of these observations may not be known until the ability to modulate surfactant levels and surfactant activity in the alveolar space is more clearly developed.

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